Difference in Plumage Color Used in Species Recognition between Incipient Species Is Linked to a Single Amino Acid Substitution in the Melanocortin-1 Receptor

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Submitted October 22, 2008; Accepted February 12, 2009; Electronically published June 2, 2009

Online enhancements: appendix tables, figure.

ABSTRACT: Many studies demonstrate that differences in mating signals are used by incipient species in recognizing potential mates or sexual competitors (i.e., species recognition). Little is known, however, about the genetic changes responsible for these differences in mating signals. Populations of the *Monarcha castaneiventris* flycatcher vary in plumage color across the Solomon Islands, with a subspecies on Makira Island having chestnut bellies and blue-black upper parts (*Monarcha castaneiventris megarhynchus*) and a subspecies on neighboring satellite islands being entirely blue-black (melanic; *Monarcha castaneiventris ugiensis*). Here we show that a single nonsynonymous point mutation in the melanocortin-1 receptor (*MC1R*) gene is present in all melanic birds from one island (Santa Ana) but absent in all chestnut-bellied birds from Makira Island, implicating this mutation in causing melanism. Birds from a second satellite island (Ugi) do not show the same perfect association between this *MC1R* variant and plumage color, suggesting an alternative mechanism for melanism on this island. Finally, taxidermic mount presentation experiments in Makira (chestnut) and Santa Ana (melanic) suggest that the plumage difference mediates species recognition. Assuming that the signals used in species recognition are also used in mutual mate choice, our results indicate that a single amino acid substitution contributes to speciation.

Keywords: speciation, species recognition, premating isolation, MC1R, *Monarcha*.

Introduction

New species arise when barriers to gene flow between taxa evolve (i.e., reproductive isolation; Dobzhansky 1937; Mayr 1942). In many organisms, reproductive isolation often starts with individuals from distinct populations failing to recognize each other as potential mates (i.e., species recognition; Baker and Baker 1990; Gray and Cade 2000; Shaw and Parsons 2002; Patten et al. 2004; Seehausen et al. 2008). Such discrimination between incipient species is typically mediated by divergent mating signals, including visual (Baker 1991; Seehausen et al. 2008), acoustic (Irwin et al. 2001; Grant and Grant 2002*a*, 2002*b*), and olfactory (Mullen et al. 2007) signals. For instance, in closely related lazuli (*Passerina amoena*) and indigo (*Passerina cyanea*) buntings, males and females use divergent song and plumage color in recognizing sexual competitors and potential mates (Baker and Baker 1990; Baker 1991). However, despite many studies establishing a clear role for divergent mating signals in speciation (reviewed in Panhuis et al. 2001; Boughman 2002), the underlying genetic changes responsible for the differences in mating signals are poorly understood, particularly in natural populations of nonmodel organisms (Coyne and Orr 2004; Price 2007).

As an example of incipient speciation on islands, Mayr (1942) discussed several populations of the *Monarcha castaneiventris* flycatcher endemic to the Solomon Islands that vary in body size and, most distinctly, in plumage color (see also Mayr and Diamond 2001; Filardi and Smith 2005; Uy et al. 2009). In particular, two subspecies endemic to islands that are a mere 8 km apart in the southeast region of the archipelago are very similar in morphology but differ strikingly in plumage color. *Monarcha castaneiventris megarhynchus* has a chestnut belly with iridescent blue-black upper parts and is endemic to Makira Island, and *Monarcha castaneiventris ugiensis* is entirely iridescent blue-black, lacking the chestnut belly, and is endemic to the smaller satellite islands of Santa Ana and Santa Catalina to the east and Ugi and Three Sisters to the north (Mayr 1942; Mayr and Diamond 2001; fig. 1). In addition to

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Am. Nat. 2009. Vol. 174, pp. 244–254.
DOI: 10.1086/600084

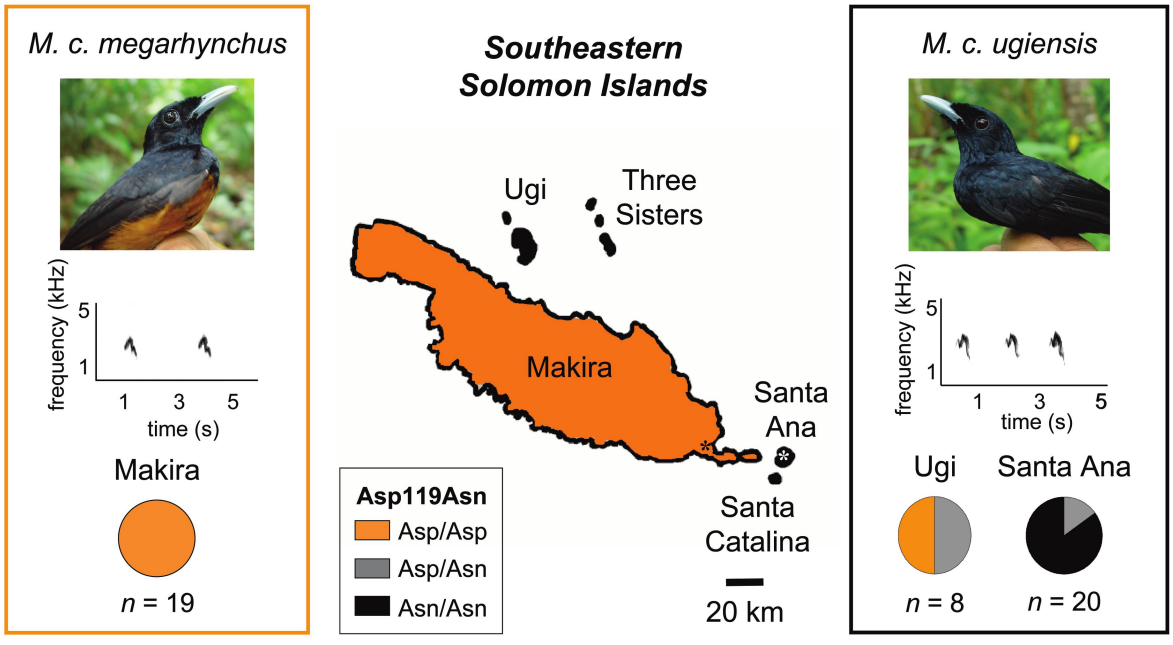

Figure 1: Distribution, plumage color, and song structure variation and *MC1R* genotype frequency (*inset pie charts*) between the chestnut-bellied and melanic *Monarcha castaneiventris* ssp. forms of southeastern Solomon Islands. The ranges of the two subspecies are color coded. *Orange*, chestnut-bellied form (*Monarcha castaneiventris megarhynchus*; Makira Island). *Black*, melanic form (*Monarcha castaneiventris ugiensis*; Santa Ana, Santa Catalina, Ugi, and the Three Sisters). The *M. c. ugiensis* sonogram shown was recorded from Santa Ana Island. Songs from Ugi Island are similar in structure. Song recordings from Santa Catalina and the Three Sisters are not available. On the basis of spectrophotometry data, Ugi and Santa Ana birds are near identical in color (J. A. C. Uy, unpublished data). Asterisks indicate sites where the mount presentation and song playback experiments were conducted.

plumage differences, the two taxa show quantitative differences in their songs (fig. 1). Phylogenetic analyses of the entire *M. castaneiventris* complex show that *M. c. megarhynchus* and *M. c. ugiensis* form a well-supported clade that is sister to the other *M. castaneiventris* subspecies; however, the two taxa are not reciprocally monophyletic as a result of the retention of ancestral polymorphisms caused by their recent divergence and/or gene flow (Uy et al. 2009; J. Poelstra and J. A. C. Uy, unpublished data). Gene flow is likely, since melanic birds have been sighted occasionally on Makira Island and chestnut-bellied birds have been sighted occasionally on the satellite islands of Ugi and Santa Ana (Mayr and Diamond 2001; J. A. C. Uy and C. E. Filardi, personal observation).

Studies across a wide range of avian and mammalian taxa show a clear association between dark coloration (i.e., melanism) and mutations in melanocortin-1 receptor (MC1R), a G protein–coupled receptor found in melanocytes that regulates melanin synthesis (e.g., Kijas et al. 1998; Våge et al. 1999; Theron et al. 2001; Nachman et al. 2003; Doucet et al. 2004; Mundy et al. 2004; Hoekstra et al. 2006). Similar changes may account for the difference in plumage color between the entirely black *M. c. ugiensis* (hereafter melanic) and the chestnut-bellied *M. c. megarhynchus* (hereafter chestnut-bellied) subspecies. Here we test for the role of divergent plumage color in the recognition of conspecifics and explore the underlying genetics of the plumage difference between the melanic and chestnut-bellied populations of Santa Ana and Makira. Given the number of studies indicating that signals used in species recognition are also used in female and/or mutual mate choice (e.g., Baker and Baker 1990; Baker 1991; Patten et al. 2004; Bernal et al. 2007), we discuss the implications of our results for the evolution of reproductive isolation between these emerging species.

Study System

Monarcha flycatchers are insectivorous leaf gleaners that inhabit the lower and middle strata of forests (Filardi and Smith 2008; Uy et al. 2009). They are socially monogamous, and breeding has been recorded throughout the year. Incidental evidence, however, suggests that individual birds do not initiate more than a single clutch during an annual cycle (C. E. Filardi, unpublished data). Pairs defend breeding or nesting territories from other breeding pairs

and are most aggressive before and during an active breeding attempt (C. E. Filardi, unpublished data). Previous and preliminary experiments indicate that territory owners from all subspecies tested respond aggressively to conspecific songs and taxidermic mounts (Filardi and Smith 2008; Uy et al. 2009; J. A. C. Uy and C. E. Filardi, unpublished data). In *Monarcha castaneiventris richardsii*, a dichromatic subspecies, males and females respond to homotypic mounts, with males often taking the lead in territory defense (Uy et al. 2009). Similarly, in the monochromatic *Monarcha castaneiventris castaneiventris* subspecies, two birds often respond aggressively to conspecific song playbacks and mount presentations, with males responding first to the simulated intrusion (as confirmed by visual inspection of gonads during mount and specimen preparation; see Uy et al. 2009; C. E. Filardi and C. E. Smith, unpublished data). These observations indicate that both sexes defend territories from conspecific intruders. Finally, mount presentation experiments in the dichromatic *M. c. richardsii* subspecies demonstrate that males preferentially attack adult male mounts and solicit matings from female mounts, indicating that plumage color is used in sexual interactions and likely in mate choice (Filardi and Smith 2008).

Recent taxonomic treatment of the *Monarcha castaneiventris* complex classifies some distinct insular populations as allospecies (e.g., the dichromatic *M. c. richardsii* as *M. richardsii* and *M. c. erythrostictus* as *M. erythrostictus*) and groups the entire Solomon endemic clade with three Australasian flycatchers to form the larger *Monarcha melanopsis* superspecies complex (Mayr and Diamond 2001). This classification, however, is not consistent with molecular data, which indicate that the entire *M. castaneiventris* complex forms a clear and separate monophyletic group and that some allospecies fail to form distinct monophyletic clades (e.g., *M. c. erythrostictus* groups with *M. c. castaneiventris*, and *M. c. castaneiventris* is paraphyletic; see Filardi and Moyle 2005; Filardi and Smith 2005; Uy et al. 2009). For simplicity, we follow Mayr's (1942) original taxonomy, which classifies the Solomon endemics as subspecies of the variable *M. castaneiventris* complex (see also Uy et al. 2009).

Material and Methods

Mount Presentation and Song Playback Experiments

To test the hypothesis that divergent plumage color and song are used in species recognition, we ran taxidermic mount presentation and song playback experiments in the melanic Santa Ana and chestnut-bellied Makira populations. This is a widely used experiment to infer the role of divergent signals in the recognition of sexual competitors or potential mates (e.g., Baker and Baker 1990; Irwin et al. 2001; Grant and Grant 2002*a*, 2002*b*; Patten et al. 2004; Balakrishnan and Sorenson 2006; Bernal et al. 2007; Seddon and Tobias 2007). For the melanic *Monarcha castaneiventris ugiensis*, we tested 60 territories in Santa Ana Island (10°50.316′S, 162°27.348′E) during June 11–19, 2007, and May 4-9, 2008. For the Makira Island chestnut-bellied *Monarcha castaneiventris megarhynchus*, we tested a total of 55 territories from May 28 to June 10, 2007, and from April 30 to May 3, 2008, in Star Harbor (10°49.120′S, 162°17.139′E) near the easternmost tip of Makira Island and ~12 km from Santa Ana Island (fig. 1).

In Santa Ana and Makira, we searched for territory owners by walking along trails and finding calling pairs. At each territory, we then randomly chose one of five treatment groups: (1) homotypic (same type) mount and song, (2) homotypic mount with heterotypic (different type) *Monarcha castaneiventris* ssp. song, (3) heterotypic *M. castaneiventris* ssp. mount with homotypic song, (4) heterotypic *M. castaneiventris* ssp. mount and song, and (5) heterospecific golden whistler *Pachycephala pectoralis* mount and song (*M. c. ugiensis*, $n = 12$ trials per treatment; *M. c. megarhynchus*, $n = 11$ trials per treatment). The golden whistler is a sympatric and ecologically similar species (i.e., both are leaf-gleaning insectivores), and so this treatment serves as a control and to test the response of *M. castaneiventris* territory owners to a heterospecific, ecological competitor. We took the global positioning system (GPS) coordinates of each territory to ensure that we did not return to the same pair for subsequent experiments. On the basis of the GPS coordinates, the nearest-neighbor distances were, on average, more than 100 m apart (mean ± SE; Santa Ana, 126.24 ± 9.52 m, range 40–396 m, $n = 60$; Makira, 132.43 ± 10.25 m, range 21–367 m, $n = 55$). For territories that were closer (<40 m), adjacent territorial birds were often heard calling during our mount presentation experiments. We are therefore confident that each trial was run in a unique territory.

Kroodsma et al. (2001) advocate an experimental protocol that uses a unique stimulus for each trial to avoid simple pseudoreplication, which is a protocol that uses a single exemplar to represent an entire class of signals. Using a new exemplar for each mount presentation trial is not feasible; however, we avoided simple pseudoreplication by using multiple exemplars per taxon and a mixed-model nested ANOVA for hypothesis testing (details below). Two adult males were caught and prepared for taxidermic mounts for each taxon (*M. c. megarhynchus* and *Pachycephala pectoralis christophori* from Makira and *M. c. ugiensis* and *P. p. christophori* from Santa Ana). The use of additional mounts may provide a better representation of the two plumage types; however, variation in plumage color between the two forms is qualitative (i.e., melanic

vs. chestnut bellies), and so the limited number of taxidermic mounts effectively represents each form. For song playbacks, we recorded long-range advertisement calls (whistles) from six different individuals for each taxon with a Marantz (Mahwah, NJ) PMD670 digital recorder set at 16-bit pulse code modulation and 48-kHz sampling rate and fitted with a Sennheiser (Old Lyme, CT) shotgun microphone. Mount and recording used for each trial were chosen randomly. In addition to whistles, two more call types are used by *M. castaneiventris*: a raspy call used during aggressive interactions and a soft chatter used during interactions in larger aggregations or courtship between sexes (Filardi and Smith 2008; Uy et al. 2009). The raspy and chatter call types are structurally similar among all subspecies, and so we used only whistles in our playback experiments. Mounts were perched on a locally collected sapling ~2 m tall and placed adjacent to vegetation suitable for perching by territorial birds. Beneath the mount's perch, we fitted a small speaker (miniamplifier; RadioShack, Forth Worth, TX) and a digital player (Ipod Shuffle; Apple, Seattle, WA), concealed by leaves collected from the habitat. After setup, the digital player played 3 min of silence before broadcasting whistles to start the experiment. On the basis of preliminary observations, territory owners attacked homotypic mounts within 2 min; hence, each trial lasted for 3.5 min (210 s). Observations were conducted ~15–20 m away from the mount by two observers who were concealed in thick vegetation. To ensure consistency in behavioral observations, all experiments were run by a single observer (J. A. C. Uy) aided by a local field guide who helped in spotting birds in the canopy or before approach. Observations were spoken into a recorder, which allowed for an accurate quantification of behavior by a single observer and recording of the vocal responses of territorial birds. All trials were run between 0630 and 1100 hours and between 1500 and 1730 hours, the time periods in which individuals were observed to sing most often from territories.

Behavioral responses were noted throughout the experiment, and we focused our analyses on behaviors that were likely assays of aggression or recognition of sexual competitors/conspecifics: (1) number of attacks or hits; (2) time spent perched on the mount's stick; (3) time spent perched on adjacent vegetation (<2 m); (4) number of flights near the mount (<2 m) without contact; (5) time spent within 2 m of the mount emitting raspy, aggressive calls; (6) time spent >2 m from the mount emitting raspy calls; (7) time spent within 2 m of the mount emitting whistles; (8) time spent >2 m from the mount emitting whistles; (9) time spent within 2 m of the mount emitting chatter calls; (10) time spent >2 m from the mount emitting chatter calls; (11) time spent calling in the canopy; and (12) total time spent in the canopy away from the mount. We used the 2-m stick on which the mount was perched as a reference for our measure of distance from the mount. Because these behavioral variables are most likely correlated, we used a principal component analysis (PCA) to collapse them into fewer orthogonal scores that characterized overall aggression or recognition (Filardi and Smith 2008; Uy et al. 2009). Using the varimax with Kaiser normalization rotation method, the PCA extracted five PC scores (eigenvalues >1) that explained 68.71% of the variation among territory holders in their response to the mount and song playbacks (PC1, 27.92%; PC2, 12.12%; PC3, 10.76%; PC4, 9.10%; PC5, 8.81%). The first PC score (PC1) was clearly associated with aggressive behavior (e.g., number of attacks; see table A1 in the online edition of the *American Naturalist*); therefore, we used PC1 as the dependent variable in our subsequent analyses.

Because territory owners typically ignored the golden whistler stimuli, we ran two separate analyses to ensure that our results were not driven by the inclusion of the golden whistler trials. First, we ran a mixed-model nested ANOVA that excluded the golden whistler trials, testing for the effects of plumage and song type (fixed factors), with specific mount and song recording (random factors) nested within the plumage and song type, respectively. This is the experimental and statistical design advocated by Kroodsma et al. (2001) and used effectively by others in playback experiments with limited number of exemplars (Grant and Grant 2002*a*, 2002*b*). Note that this is a conservative test that completely avoids simple pseudoreplication. Other studies have alternatively tested for a specific mount or recording effect and, after not finding an effect, pooled their data set and tested for treatment effects (Patricelli et al. 2002; Chaine and Lyon 2008). The effects of plumage type were far stronger when we used this type of analysis (see table A2 in the online edition of the *American Naturalist*). However, to completely avoid pseudoreplication, we present Kroodsma et al.'s (2001) more conservative protocol in the main text.

Second, we ran a mixed-model ANOVA that included the golden whistler trials. However, because the inclusion of the golden whistler trials resulted in an unbalanced experimental design (e.g., no combination of homotypic *M. castaneiventris* plumage and golden whistler song treatment), we could not run a similar nested ANOVA and instead used the five treatment groups as the independent factor and PC1 as the dependent variable. Note that in this instance, each stimulus for each trial is unique (i.e., unique combination of taxidermy mount and song recording), and so pseudoreplication is not an issue. We then ran a linear contrast test for the main effect of the five treatment groups. The linear contrast was constructed using the following coefficients (1, 0.5, 0, −0.5, −1), which corresponded to the five treatment groups in the following

order (homotypic mount and song, homotypic mount and heterotypic *M. castaneiventris* song, heterotypic mount and homotypic song, heterotypic mount and song, and golden whistler mount and song). A significant linear relationship, therefore, indicates that the intensity of response declines linearly. In addition, we ran corrected post hoc pairwise comparisons (Fisher's least significant difference) to test for differences between treatments. Because our data set may not meet the assumptions of parametric tests, we also used randomization tests to calculate probability values for hypotheses testing for all analyses (i.e., compared the *F* statistics with a randomized distribution based on our data set; Cassell 2002). Results from randomization and parametric tests were near identical, and we present both in table 1. Statistical analyses were performed using SAS (ver. 8.1; SAS Institute, Cary, NC). All tests of hypotheses are two-tailed.

Table 1: Mixed-model nested ANOVA of aggressive response (principal component 1) by territory owners to taxidermic mount presentations and call playbacks, excluding the heterospecific golden whistler control

Factor	df	Type III SS	*F*	*P*
Plumage type	1, 1.34[a]	15.84	80.89	.037 (.045)
Mount	2, 75	.46	.29	.751 (.733)
Call type	1, 9.56[a]	2.19	3.72	.084 (.079)
Recording	10, 75	5.91	.75	.678 (.668)
Taxon	1, 75	2.90	3.67	.059 (.059)
Plumage × call	1, 75	1.46	1.80	.178 (.190)
Residuals	75	59.24		

Note: Probability values (*P*) from parametric ANOVA and randomization tests (in parentheses) are provided.

[a] Satterthwaite-corrected degrees of freedom.

Sequencing and Sequence Analyses

We sequenced ~810 base pairs (bp) of the coding region of the *MC1R* gene corresponding to amino acids 25–295 of the chicken MC1R following a revised protocol for birds (Cheviron et al. 2006). All studies that have established a link between *MC1R* mutations and melanic plumage found substitutions within this region (Theron et al. 2001; Doucet et al. 2004; Mundy et al. 2004; Baião et al. 2007). We sequenced 20 melanic individuals from Santa Ana Island, eight melanic individuals from Ugi Island, and 19 chestnut-bellied individuals from Makira Island. We also sequenced *MC1R* for individuals from the three other *M. castaneiventris* subspecies and a closely related species, *Monarcha cinerascens*, which serves as the outgroup (Filardi and Smith 2005; Uy et al. 2009). In addition, we sequenced 903 bp of the mitochondrial nicotinamide adenine dinucleotide dehydrogenase (*ND2*), and two nuclear introns, 504 bp of transforming growth factor β-2 (*TGFβ2*), and 704 bp of myoglobin intron 2 (*Myo2*) for individuals from Santa Ana and Makira (for details on sampling, see table A3 in the online edition of the *American Naturalist*). For these markers, we used protocols developed for birds (Primmer et al. 2002; Filardi and Moyle 2005; Filardi and Smith 2005). Additional nuclear and mitochondrial loci were sequenced for the Santa Ana and Makira populations because these were the sites where we ran our behavioral experiments. Sequences were aligned using the program Sequencher 4.8 (Gene Codes, Ann Arbor, MI). We inferred haplotype phase for the three nuclear genes using the program PHASE (Stephens et al. 2001). Haplotypes with low phasing support (i.e., posterior probability <0.90) were cloned and sequenced to confirm correct phasing. Population differentiation (F_{st}) indices and gene flow estimates were calculated using Arlequin (ver. 2.0; Schneider et al. 2000) and Isolation with Migration (IM; Hey and Nielsen 2004), respectively.

For the Santa Ana and Makira populations, we used the *ND2*, *Myo2*, and *TGFβ2* sequence data in the program IM to simultaneously estimate six population genetic parameters, which were all scaled to the neutral mutation rate μ: $\theta_{melanic}$ (population mutation rate of the melanic Santa Ana population), $\theta_{chestnut}$ (population mutation rate of the chestnut-bellied Makira population), $\theta_{ancestral}$ (population mutation rate of the ancestral population), t (time since divergence), $m_{melanic \rightarrow chestnut}$ (migration rate from the melanic into the chestnut-bellied population), and $m_{chestnut \rightarrow melanic}$ (migration rate from the chestnut-bellied into the melanic population; Hey 2005). We performed initial IM runs with large, flat priors for each parameter (Won and Hey 2005). On the basis of the results of these preliminary runs, we defined narrower upper bounds for each prior that encompassed the entire posterior distribution for each parameter estimate (see table A4 and fig. A1 in the online edition of the *American Naturalist*). These upper bounds were used to define uniform prior distributions for each parameter. The final analysis was allowed to run for 50,000,000 steps following a 1,000,000-step burn-in. The effective sample size for each parameter was at least 100.

IM assumes no recombination within loci. We tested for recombination using the four-gamete test in DnaSP (ver. 4.10; Rozas et al. 2003). Recombination was detected in both *Myo2* and *TGFβ2*. For these loci, we used the largest nonrecombining block of contiguous sequence in the IM analysis. Using the parameters estimated from the final IM run (table A4), we calculated effective number of gene migrants following Hey (2005): (1) effective number of gene migrants from melanic Santa Ana into chestnut-bellied Makira $= \theta_{chestnut} m_{melanic \rightarrow chestnut}/2$; (2) effective number of gene migrants from chestnut-bellied Makira into melanic Santa Ana $= \theta_{melanic} m_{chestnut \rightarrow melanic}/2$.

Results and Discussion

Plumage Divergence and Species Recognition

We conducted taxidermic mount presentation and song playback experiments to determine whether the differences in plumage color and song structure are indeed used in species recognition between the chestnut-bellied birds of Makira and the melanic birds of Santa Ana. For both populations, territory owners consistently ignored the golden whistler stimuli and responded most aggressively to homotypic plumage and song, least aggressively to heterotypic plumage and song, and intermediately to a mismatch in song and plumage (fig. 2). To ensure that our results are not driven by the lack of response to the golden whistler stimuli, we first excluded the golden whistler trials from our analysis. A mixed-model nested ANOVA suggests that plumage type influences the intensity of aggressive response in both flycatcher populations, with song type showing a statistical trend (tables 1, A2). Further, in the 65 trials where territory owners approached the taxidermic mounts, every trial evoked a strong response from at least two territorial birds, with one individual taking the lead (e.g., approaching first). These birds are monochromatic, and so sex identification during the experiments was not possible. *Monarcha* flycatchers, however, are socially monogamous (Filardi and Smith 2008), and in the dichromatic taxon *Monarcha castaneiventris richardsii*, males and females often respond to conspecific taxidermic mounts (Uy et al. 2009). The territorial pairs that responded to our experiment were thus likely breeding pairs, suggesting that both males and females use divergent color in species recognition.

The golden whistler stimulus serves as a control to assay the general response of *Monarcha castaneiventris* to a sympatric, ecological competitor, and the lack of response to the golden whistler is consistent with the hypothesis that in *M. castaneiventris*, the strong response to homotypic signals is a response to a sexual rather than an ecological competitor (as in Uy et al. 2009). Inclusion of the golden whistler trials in our analysis shows a strong treatment effect (i.e., plumage and song type, melanic Santa Ana: $F_{4,55} = 8.70$, Type III SS = 24.34, $P < .001$; chestnut-bellied Makira: $F_{4,50} = 8.27$, SS = 19.18, $P < .001$). Further, a linear contrast for the treatment main effect, which tests for a declining linear relationship among the responses to the five treatment types (as ordered in fig. 2), accounted for >95% of the variation explained by the treatment main effect (melanic: $F_{1,55} = 33.55$, SS = 23.46, $P < .001$; chestnut-bellied: $F_{1,50} = 31.83$, SS = 18.46, $P < .001$). The linear contrast therefore shows that the intensity of response to the heterotypic stimuli is intermediate to the responses to the homotypic and golden whistler stimuli (note that the significant linear contrast remains strong even when the golden whistler control is excluded from the analyses; see table A5 in the online edition of the *America Naturalist*). Similarly, corrected post hoc pairwise comparisons indicate a declining pattern (fig. 2). These results indicate that the stronger response to homotypic stimuli is most likely a response to sexual rather than ecological competitors. Further, the response to homotypic mounts with heterotypic songs is intermediate to the responses to homotypic mount with homotypic song and to heterotypic mount with homotypic song, suggesting that song, to a limited extent, may also influence the recognition of sexual competitors.

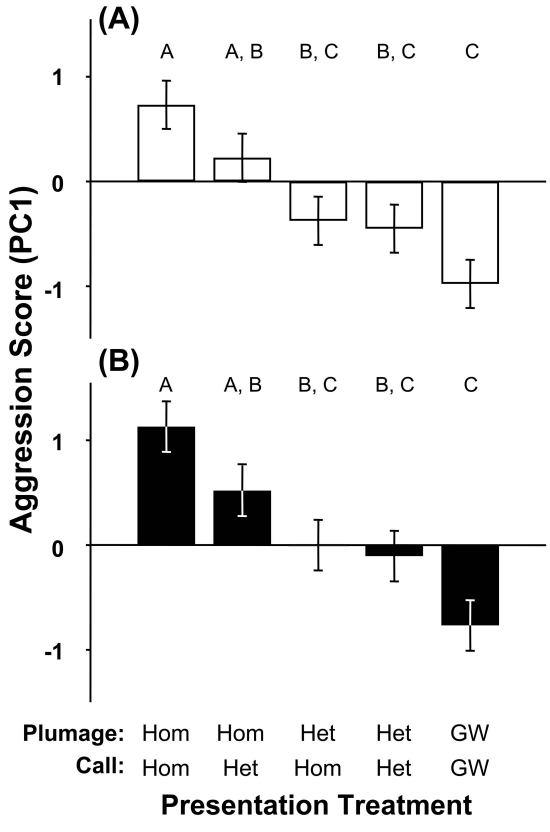

Figure 2: Response by territory owners to taxidermic mount presentation and song playback experiments. Mean (±SE) aggression scores (principal component [PC] 1) of Makira Island chestnut-bellied (*A*; $n = 11$ trials per treatment; *open bars*) and Santa Ana Island melanic (*B*; $n = 12$ trials per treatment; *solid bars*) territory owners toward various combinations of plumage and song types (*Hom* = homotypic, *Het* = heterotypic, *GW* = golden whistler control). Aggression scores were derived from PC analysis using the observed behavioral responses of territory owners (table A1 in the online edition of the *American Naturalist*). Positive scores indicate more intense aggressive response to the plumage and song types (conversely, negative scores indicate lack of response). The SEs shown are the least squares estimates of standard error. Different letters above each treatment indicate significant pairwise differences (Fisher's least significant difference; see "Material and Methods").

Genetics of Plumage Differences

With evidence suggesting that divergent plumage color may mediate species recognition between melanic Santa Ana and chestnut-bellied Makira birds, we explored the underlying genetic changes that may be responsible for the difference in plumage color between these populations by sequencing most of the coding region of *MC1R*. We found three polymorphisms at nucleotide sites 237, 355, and 441 (aligned with chicken *MC1R*; fig. 3). Only the point mutation at site 355, however, is a nonsynonymous substitution, resulting in a change from aspartic acid (Asp) to asparagine (Asn) at amino acid 119 (Asp119Asn). Strikingly, melanic birds from Santa Ana Island were either homozygous ($n = 17$) or heterozygous ($n = 3$) for the Asn^{119} allele, whereas all chestnut-bellied birds from Makira Island ($n = 19$) were homozygous for the Asp^{119} allele (Fisher's exact test, $P \ll .001$; fig. 1). Further, we sequenced *MC1R* in birds with chestnut bellies from the three other major *M. castaneiventris* color forms (*Monarcha castaneiventris castaneiventris*, $n = 11$; *M. c. richardsii*, $n = 6$; *Monarcha castaneiventris erythrostictus*, $n = 2$) and the outgroup *Monarcha cinerascens* ($n = 1$) and found that all individuals were homozygous for the Asp^{119} allele. These results suggest that the Asn^{119} variant is derived from the ancestral Asp^{119} allele (fig. 3). But, more importantly, the clear and statistically significant association between the *MC1R* variants and plumage color strongly suggests that the Asp119Asn mutation contributes to the expression of the melanic plumage of Santa Ana birds and appears to be dominant, since heterozygous individuals are melanic in plumage (e.g., Kijas et al. 1998; Theron et al. 2001; Våge et al. 2003).

Three lines of evidence further support a causal association between the Asn^{119} mutation and melanism. First, breeding experiments indicate that the identical amino acid substitution at the homologous site cosegregates with melanism in several strains of sheep (Våge et al. 1999, 2003) and pigs (Kijas et al. 1998). Second, amino acid 119 is in the third transmembrane of MC1R and part of a negatively charged pocket that is crucial for binding with the positively charged asparagine residue of its agonist (Lu et al. 1998). Functional in vitro mutagenesis experiments in mouse MC1R show that mutations in position 119 and adjacent amino acids can alter the negative charge of the third transmembrane pocket, resulting in the reduction of agonist binding affinity and the potential activation of MC1R by mimicking agonist binding (Våge et al. 1997; Lu et al. 1998). Finally, one mitochondrial and two other nuclear markers show only weak population structure (mitochondrial gene: *ND2*, fixation index or $F_{st} = 0.00$, $P = .42$; nuclear introns: *Myo2*, $F_{st} = 0.101$, $P < .01$; *TGFβ2*, $F_{st} = 0.092$, $P < .01$), whereas the *MC1R* alleles show very strong population differentiation ($F_{st} = 0.848$, $P < .001$). These results suggest that lineage sorting is an unlikely explanation for the perfect association between the Asp119Asn mutation and plumage color between Santa Ana and Makira birds.

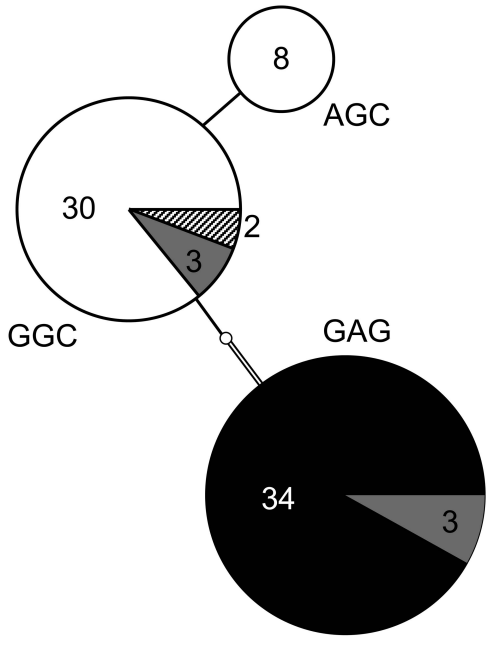

Figure 3: *MC1R* haplotype network derived with TCS (Clement et al. 2000) for individuals from the islands of Santa Ana (melanic) and Makira (chestnut bellied) and the outgroup species *Monarcha cinerascens* (chestnut bellied). Haplotype phasing indicated three *MC1R* haplotypes: GGC, AGC, and GAG (corresponding to nucleotide sites 237, 355, and 441, respectively). Black (homozygotes) and gray (heterozygotes) correspond to melanic Santa Ana individuals, white corresponds to chestnut-bellied Makira individuals, and hatched lines correspond to *M. cinerascens*. On the basis of comparison with the outgroup species, the most likely ancestral state for the *MC1R* is the GGC haplotype. Numbers indicate number of alleles. The double line represents the nonsynonymous substitution, while single lines indicate synonymous substitutions.

Additional population genetic analyses of *ND2* and the two nuclear introns using the program IM suggest contemporary gene flow between the Santa Ana and Makira populations, with asymmetrical introgression from the melanic to the chestnut-bellied form (effective number of gene migrants from melanic Santa Ana to chestnut Makira, 37.07; from Makira to Santa Ana, 0.30; see fig. A1). These estimates from the IM analyses, however, should be interpreted with caution, since violation of isolation-migration model assumptions may lead to biased parameter estimates (Becquet and Przeworski, forthcoming). For instance, the isolation-migration model assumes no geographic structure in the ancestral population, and when this assumption is violated, IM can overestimate ancestral

effective population size and provide spurious support for contemporary gene flow (Becquet and Przeworski, forthcoming). Because we cannot explicitly test the assumptions of the isolation-migration model for our data set, our parameter estimates may be less reliable. However, our results showing very different F_{st} values among loci coupled with observations of contact between melanic and chestnut-bellied birds corroborate the possibility of gene flow between Santa Ana and Makira.

Melanic birds are also found on the satellite islands of Ugi and Three Sisters, which are situated 10 km off the northern coast of Makira and ~100 km from Santa Ana (fig. 1). Although we did not run behavioral experiments in these populations, we tested the hypothesis that identical substitutions in *MC1R* may mediate melanic coloration on Ugi Island. Melanic birds from Ugi do not show the same association between the Asn^{119} variant and melanic plumage. *MC1R* sequences of eight birds from Ugi show that four individuals were heterozygous for the derived and ancestral alleles, while the remaining four were homozygous for the ancestral allele (fig. 1). At first, these results may suggest that the clear association between the derived Asn^{119} allele and melanism in Santa Ana birds is spurious. However, the perfect and statistically robust association between this *MC1R* variant and melanic birds in Santa Ana and the striking convergence between Santa Ana birds and distantly related sheep and pigs strongly support a causal relationship between the Asn mutation and melanic coloration. Our results, therefore, suggest that birds from Ugi Island may have evolved an additional mechanism for melanism. This is similar to situations found in rock pocket mice (Nachman et al. 2003) and beach mice (Hoekstra et al. 2006; Steiner et al. 2008), where some populations show a clear association with *MC1R* variants and melanism while others do not. One possibility is that mutations in genes that interact with (Rieder et al. 2001; Anderson et al. 2009) or regulate (Steiner et al. 2007) *MC1R* have since arisen in the Ugi Island population, making mutations in *MC1R* no longer necessary to express the melanic phenotype and allowing the ancestral *MC1R* allele to increase in frequency through drift or introgression from Makira Island. Preliminary population genetic analyses of gene flow between the islands of Ugi, Santa Ana, and Makira indicate limited gene flow between Santa Ana and Ugi, much smaller than that between Santa Ana and Makira (J. W. Poelstra and J. A. C. Uy, unpublished data). This suggests that the two melanic populations may be evolving independently. Additional population and evolutionary genetic analyses should provide clear answers for the underlying genetics of Ugi Island melanic birds.

Selection for Melanism

Given the molecular evidence for gene flow in mitochondrial and nuclear intron loci and observations of occasional contact between birds from Santa Ana and Makira (Mayr and Diamond 2001; J. A. C. Uy and C. E. Filardi, personal observation), recognition by both sexes based on divergent plumage color provides a mechanism for the differentiation in *MC1R* and maintenance of plumage differences between Santa Ana and Makira. The possible mechanism(s) that favored the fixation of the melanic phenotype in Santa Ana and other satellite islands, however, remains unknown. Several studies indicate that biotic (Burtt and Ichida 2004; Anderson et al. 2009) and abiotic (Theron et al. 2001) factors can favor melanism, and we discuss some of these possibilities below.

First, melanism may be linked to other traits that provide advantages to melanic birds, especially during colonization of and establishment in novel habitats. Our analysis revealed a nearly significant difference in response to the mount presentation and call playback experiments between melanic Santa Ana and chestnut-bellied Makira birds (table 1). This difference is in the overall intensity of response across all treatment types, with melanic birds being more aggressive than their chestnut-bellied counterparts (i.e., higher overall PC1 values across treatments; fig. 2). The satellite islands are about 100 times smaller than Makira and so could have fewer available breeding territories. Hence, the more aggressive melanic birds may be more successful than the chestnut-bellied birds in securing breeding territories on the smaller islands. The association between aggression and melanism may be mediated by a shared biochemical pathway for the expression of melanic coloration and aggressive behavior (see Hadley 1996) and has been similarly observed in other melanic organisms (e.g., mosquitofish; Horth 2003).

Second, natural selection may directly favor unique coloration on different islands. Because the efficacy of plumage signals is dictated by the ambient light that illuminates the signal and the background against which the signal is viewed, differences in the visual habitat between islands may select for divergent colors that best fit each habitat (e.g., Uy and Stein 2007). Melanic birds are iridescent blue-black in color, and so their plumage would be most effective in habitats rich in short-wavelength light (e.g., woodland shade with light coming from the sky). Chestnut, on the other hand, reflects long wavelengths, and so their plumage would be most effective in habitats rich in long-wavelength light (e.g., small gaps in thick forests with light coming directly from the sun; Endler 1993). This type of difference in visual habitats between islands is a distinct possibility, since satellite islands harbor forests that are more open and shorter in canopy (J. A. C. Uy, personal

observation), which should be richer in short-wavelength light (Endler 1993). In addition to variable visual habitats, natural selection by visual predators and feather-degrading microbes may also favor melanism on satellite islands. Melanic plumage is generally less conspicuous than chestnut plumage; hence, melanic birds may be less susceptible to visual predators (e.g., goshawks). However, Makira and the satellite islands do not differ in avian predator composition (Mayr and Diamond 2001), but their relative abundances have yet to be estimated. Recent studies also indicate that melanic feathers resist feather-degrading microbes better than nonmelanic feathers (Burtt and Ichida 2004). If feather-degrading microbes are more abundant and/or more intense in the satellite islands, then these microbes may select for melanic plumage.

Finally, novel mating preferences for melanic plumage may have driven the fixation of melanic color in the satellite islands (i.e., divergent sexual selection). This preference may have arisen randomly or may be linked to variable visual habitats, since selection by the visual environment may bias mating preferences for signals that are most effective in their unique habitats (e.g., Seehausen et al. 2008). In the dichromatic subspecies *M. c. richardsii*, plumage color is used in sexual interactions (Filardi and Smith 2008), suggesting that plumage may be used in mutual mate choice in this complex. Novel mating preferences and divergent sexual selection is therefore a possible mechanism that favored the fixation of the melanic phenotype in the satellite islands. Ongoing long-term research in this complex is testing these and other potential mechanisms that favor divergent coloration.

Implications for Speciation

Species may exhibit color polymorphisms that do not contribute to reproductive isolation and speciation (e.g., Nachman et al. 2003; Hoekstra et al. 2006); however, a critical point that differentiates a stable color polymorphism and the patterns we document here is the evidence for incipient reproductive isolation between forms or incipient species (see Seehausen et al. 1999; Gray and McKinnon 2007). Our comparison between the Makira Island and the Santa Ana Island populations indicates that a single point mutation is perfectly associated with a large phenotypic change that mediates species recognition between chestnut-bellied and melanic flycatchers (fig. 2). Although mating trials would be a more direct test of premating reproductive isolation, an aggressive response to taxidermic mounts and song playbacks has been used widely as an indirect and alternative assay (e.g., Ratcliffe and Grant 1983; Baker 1991; Irwin et al. 2001; Grant and Grant 2002*a*, 2002*b*; Patten et al. 2004; Balakrishnan and Sorenson 2006; Seddon and Tobias 2007; Uy et al. 2009). In fact, several studies have confirmed that traits used by territory owners in species recognition are indeed used by females in mate choice (e.g., Baker and Baker 1990; Baker 1991; Patten et al. 2004; Bernal et al. 2007; but see Searcy and Brenowitz 1988). Therefore, assuming that signals used in species recognition are also used in female or mutual mate choice (e.g., Irwin et al. 2001; Grant and Grant 2002*a*, 2002*b*; Balakrishnan and Sorenson 2006), our results suggest that the difference in plumage color between the melanic and chestnut-bellied populations of Santa Ana and Makira may result in incipient premating reproductive isolation despite gene flow between the two.

Many avian species show intraspecific variation in plumage color across islands (e.g., Mayr 1942; Bartle and Sagar 1987; Ryan et al. 1994), including cases of melanism (e.g., *Rhipidura* fantails: Atkinson and Briskie 2007; island thrush: Jones and Kennedy 2008) and more specific cases establishing a link between *MC1R* substitutions and melanism (bananaquit: Theron et al. 2001; fairy-wrens: Doucet et al. 2004; blue-footed boobies: Baião et al. 2007). In addition, melanism on islands is documented in other taxa (e.g., Senegalese grasshopper: Ritchie 1978; snakes: Nilson and Andrén 1981; lizards: Cirer and Martinez-Rica 1990; spiders: Tso et al. 2002). Although the explicit mechanisms favoring melanism on islands are yet to be conclusively or experimentally shown (but see Theron et al. 2001), melanism on islands seems to be common, indicating its general importance in island diversification. Our results suggest that a simple genetic change before or during colonization events followed by strong social selection (e.g., assortative mating) once established on islands may help explain the striking endemism of many island fauna.

Acknowledgments

We thank the people of the Solomon Islands, especially the villages of Gupuna, Marou, Marione, and Namugha, for their hospitality. For help in the field, we thank J. Murray and J. Murray of Sanbis Lodge; A. Murray and family of Star Beach Lodge; O. Masina, H. Pirigua, and family of Gupuna; M. Hemmer of South Pacific Oil; and the Makira Community Conservation Foundation. Permission to work in the Solomon Islands was granted by the Ministry of Education, Solomon Islands. For assistance in the lab, we thank C. Low and T. Pope. For help with the molecular and data analyses, we thank D. Althoff, J. Poelstra, D. C. Presgraves, K. Segraves, and W. T. Starmer. We thank R. Brown, C. Low, S. Pitnick, D. C. Presgraves, W. T. Starmer, members of the Uy lab, and two anonymous reviewers for helpful comments on this manuscript. This research adhered to the Institutional Animal Care and Use Committee guidelines for the use of vertebrate animals in research and the legal requirements of the country in

which the work was carried out. This work was funded by a National Science Foundation CAREER grant to J.A.C.U., a grant from the University of Kansas General Research Fund to R.G.M., and grants from Conservation International, the F. M. Chapman Memorial Fund, and L. C. and L. J. Sanford Funds of the American Museum of Natural History to C.E.F.

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